

Supplementary Materials and Methods

Cell lines and Cell Culture. Dissociated tissue or sphere cells (GBM146, 157, 205, 206, 218, f16w and 1105A) were cultured in Dulbecco's modified Eagle's medium (DMEM)/F12 medium supplemented with B27 (Gibco), 20 ng/mL bFGF (Peprotech), 50 ng/mL EGF (Peprotech), 100U/mL penicillin/streptomycin (Gibco), L-glutamine (Invitrogen) and 5 µg/mL heparin (Sigma-Aldrich), as described previously (19). Briefly, Heparin, bFGF, and EGF were added twice a week. Every 7-14 days, spheres were passaged into fresh media following either enzymatic dissociation with TrypLEExpress (Gibco) or chopping.

GBM1600 and GBM2313 were kindly provided from Dr. Paul Mischel's laboratory at UCLA and their short-term cultures (the number of passages within 20) were maintained in DMEM/F12 with 10%FCS. Normal human astrocytes that overexpress E6, E7, and hTERT (NHA) were obtained from the American Type Culture Collection and maintained in DMEM/F12 with 10%FCS. Primary cultures of human astrocytes were established by culturing the fetal neurospheres (f16w) with DMEM/F12 with 10%FCS for 2 weeks. Expression of GFAP in virtually all cells was confirmed by immunocytochemistry prior to the experiments.

Immunocytochemistry. Immunocytochemistry was performed as described previously (19). The following primary antibodies were used: human specific Nestin (1:200, Millipore, clone10C2), CD133 (1:1000, Abcam, ab19898), Sox2 (1:200, Millipore, mab4343), TuJ1 (1:500, Berkeley Antibodies), GFAP (1:1,000, Dako Cytomation) and Telomerase (1:200, Abcam, ab32020). Primary antibodies were visualized with Alexa Fluor 555 or 488 -conjugated secondary antibodies (Invitrogen). Hoechst 33342 was used as a fluorescent nuclear counterstain. In all experiments, specific labeling of the antibodies was confirmed by the negative control samples without primary antibodies.

Flow cytometry analysis. Flow cytometry was performed as described previously (20). Briefly, spheres were collected after treatments, dissociated into single cells and fixed by 1% paraformaldehyde. Cells were then incubated with 10 μ L of CD133-APC conjugated antibody (293C3, Milteny Biotec Inc.) for 10 min at 4 °C, followed by analysis with flow cytometry using a FACS Calibur flow cytometer (BD Biosciences). The data were analyzed by FlowJo 7.6.1 software. Three independent assays were performed for confirmation.

Apoptosis assay. Apoptosis Detection kit by fluorescent conjugated Annexin V and propidium iodide staining (Invitrogen) was used for detection of relatively

early apoptotic cell death according to the manufacturer's protocol.

Western blot. Western blot was performed as described previously (20). The primary antibodies used in this study are p53 (1:200, Santa Cruz, sc126), Telomerase (1:1000, Abcam, ab32020), c-Myb (1:2000, Abcam, ab45150), and GAPDH (1:1000, Cell Signaling, 14C10).

RT-PCR. RT-PCR was performed as described previously (20).

Transient transfection. Transfection of siRNA for GBM30 was performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol as described previously (20). Two siRNAs for c-Myb and one siRNAs for green fluorescent protein (GFP) were chemically synthesized according to Ambion's protocol and the target sequences were as follows. c-myb siRNA antisense #1,

AACACTTCAAGTTGACTTGGGCCTGTCTC; c-myb siRNA sense #1,

AACCCAAGTCAACTTGAAGTGCCTGTCTC; c-myb siRNA antisense #2,

AATGCTATCAAGAACCACTGGCCTGTCTC; c-myb siRNA sense #2,

AACCAGTGGTTCTTGATAGCACCTGTCTC; siGFP: sense, 5'-

AACTCGATGTTGTGGCGGATCCCTGTCTC -3'; antisense, 5'-

AAGATCCGCCACAACATCGAGCCTGTCTC -3'.

Lentivirus-mediated shRNA for c-Myb

A lentivirus construct to deliver c-Myb shRNA was purchased from Sigma and used according to the manufacturer's protocol. The sequence for c-Myb shRNA was

CCGGGCTCCTAATGTCAACCGAGA ACTCGAGTTCTCGGTTGACATTAGGAG
CTTTTTG.

Xenograft. All the animal experiments were approved by Institutional Animal Care and Use Committee following NIH guidelines using athymic nu/nu mice (NCI/NIH). *Ex vivo* experiment: Transplantation of GBM cells were performed as described previously (18(16)and see SI for a detail). *In vivo* experiment: GBM157 spheres were dissociated into single cells and transplanted into mouse brains as described above. Five μ L solutions containing TMS (5 pmol, 50 pmol, or 2.5 nmol) or DMSO diluted in PBS were injected into the same location with the same procedure, respectively.

Tumor size measurement. All brains were cut in a uniform fashion (20 μ M coronal brain section by cryostat), keeping every 10th section for size analysis. After immunohistochemical staining with human specific Nestin or Vimentin, photo image files that contained section with largest and/or nearest tumors to the injection tract were taken with Olympus microscope. These images were

imported into ImageJ 1.42 software and the color channels were divided into blue, green, and red ones. The blue channel images were selectively processed to convert into black and white images. The same software automatically measured intensities for immunoreactivity corresponding to the tumor size were automatically.

in situ hybridization (iFISH). iFISH assay was performed as described previously (36,3723). Briefly, undifferentiated or differentiated GBM cells were seeded on poly-L-lysine-coated coverslips and treated with 0.5 or 1.0 μ M TMS for 96 hours under the same serum-free condition. These cells were fixed with 2% paraformaldehyde/PBS and permeabilized with 0.5% Nonidet P-40/PBS. The fixed cells were incubated with 300 ng/ml Cy3-labeled peptide nucleic acid (PNA) probe, (CCCTAA)₃, in PNA dilution buffer consisting of 70% formamide, 10 mM Tris-HCl, pH7.5, 0.05% blocking reagent (Roche, cat. #1096176, Basel, Switzerland) at 83°C for 4 min and at room temperature for 2 h. After washing, the cells were blocked in PBS containing 1% BSA for 15 min and incubated with rabbit anti-53BP1 (1:100, Cell Signaling) for 2 hour. This primary antibody was detected with fluorescein-conjugated donkey anti-rabbit immunoglobulin (1:25, GE Healthcare). DNA was stained with 0.2 μ g/mL 4,6-diamino-2-phenylindole

(DAPI). Images were acquired using an Olympus IX-71 microscope with a DP70 digital camera and a Lumina Vision software (Mitani Corporation, Tokyo, Japan). For quantitative analysis, cells with more than two punctate 53BP1 foci were classified as the 53BP1 focus-positive cells.

Supplementary Figure Legends

Fig. S1.

A, Dose dependent curves of TMS effect on growth of cell lines derived from gliomas in the panel of JFCR39. **B**, Relative cell numbers in 2 patient-derived serum-propagated GBM cell cultures treated with varying doses of TMS for 96 h. **C**, The number of neurospheres (NS) derived from 5 GBM patient cells with varying doses of TMS for 14 days. Representative pictures of GBM157-derived NS with TMS at indicated doses. Original magnification; x40. NS; neurosphere. **D**, Relative cell numbers of primary astrocytes and NHA cell line overexpressing hTERT, E6, and E7 with treatment of varying doses of TMS for 96 h. Asterisk indicates statistical significance by ($*p < 0.05$). Abbreviation; M = molar.

Fig. S1

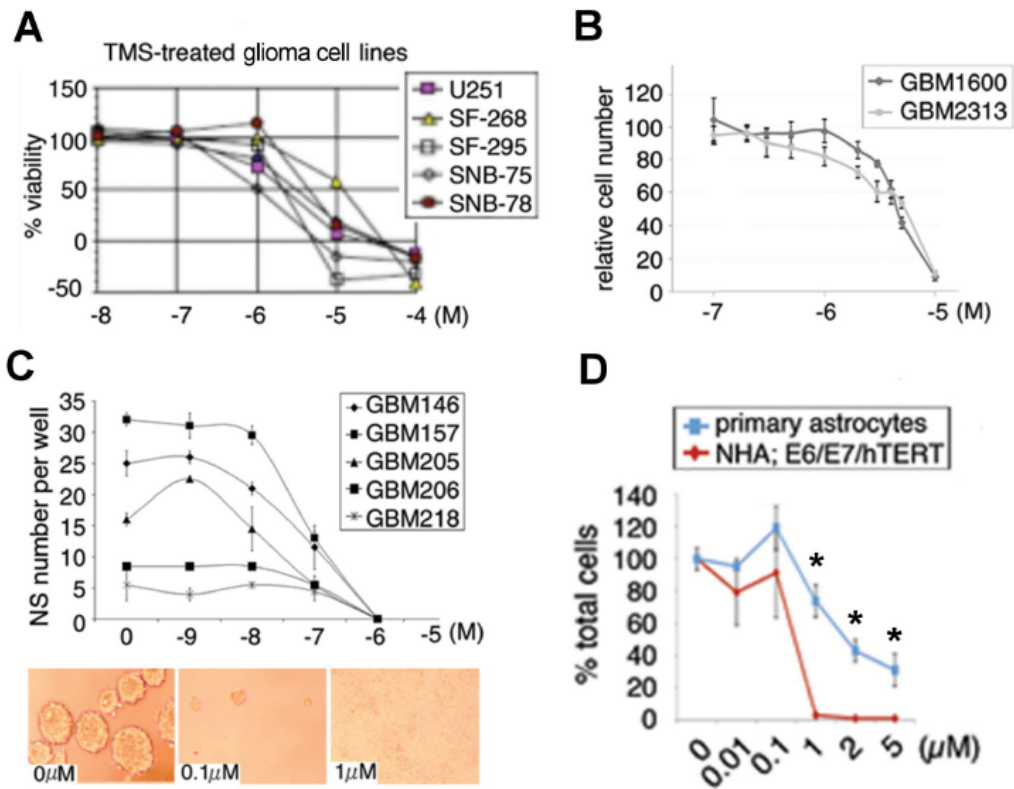


Fig. S2.

Panels of **A** and **C** indicate the character of tested cells (three GBM cell lines: GBM146, 157 and 206) and two normal neural precursors (f16w and 1105A) in serum-free medium. Panels of **B** and **D** indicate the character of the cells after 7days treatment with serum (10%FCS)-containing medium. Indicated antibodies and Hoechst dye were used as cell type-specific markers and for nuclear staining, respectively. Original magnification; x40.

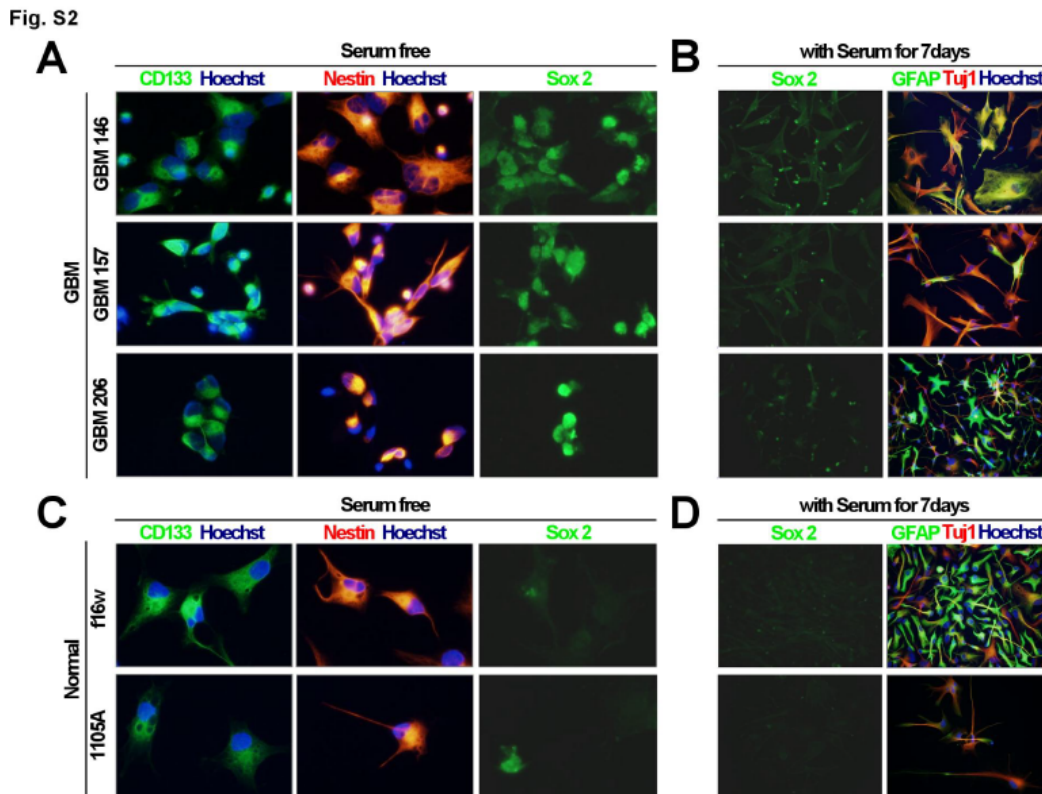


Fig. S3.

A, Histograms indicating the proportions of CD133(+) cells in GBM spheres (a; GBM157, b; GBM206) after treatment with TMS for 48 h. **B**, The graph indicates the proportion of sphere-forming cells in DMSO-treated or TMS-treated GBM157 cells following separation into CD133(+) and CD133(-) cells by cell sorting. Over 90% of sphere-forming cells were CD133(+) cells in the DMSO wells.

Fig. S3

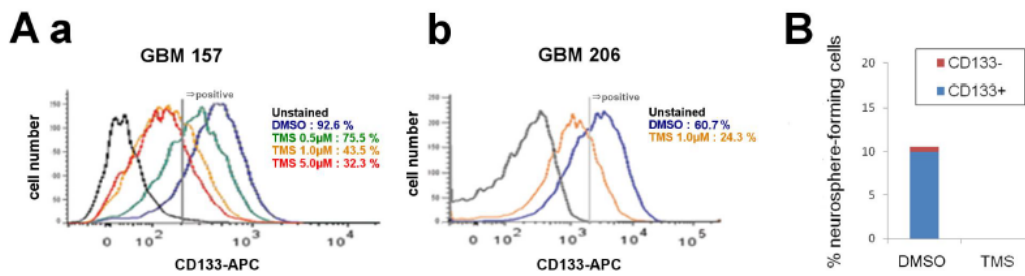


Fig. S4.

A, Schema describing the experimental flow. **B-G**, Representative pictures (left panels) indicate human specific Nestin staining (B, C, D) and Vimentin staining (E, F, G) of immunocompromised mouse brains bearing GBM sphere-derived tumors with or without TMS pre-treatment at indicated doses for 24 h. Original magnification; x2. Graphs (right panels) indicate the average of overall immunostaining intensities in each group. Asterisk indicates statistical significance. Exact p values are indicated in the figure.

Fig. S4

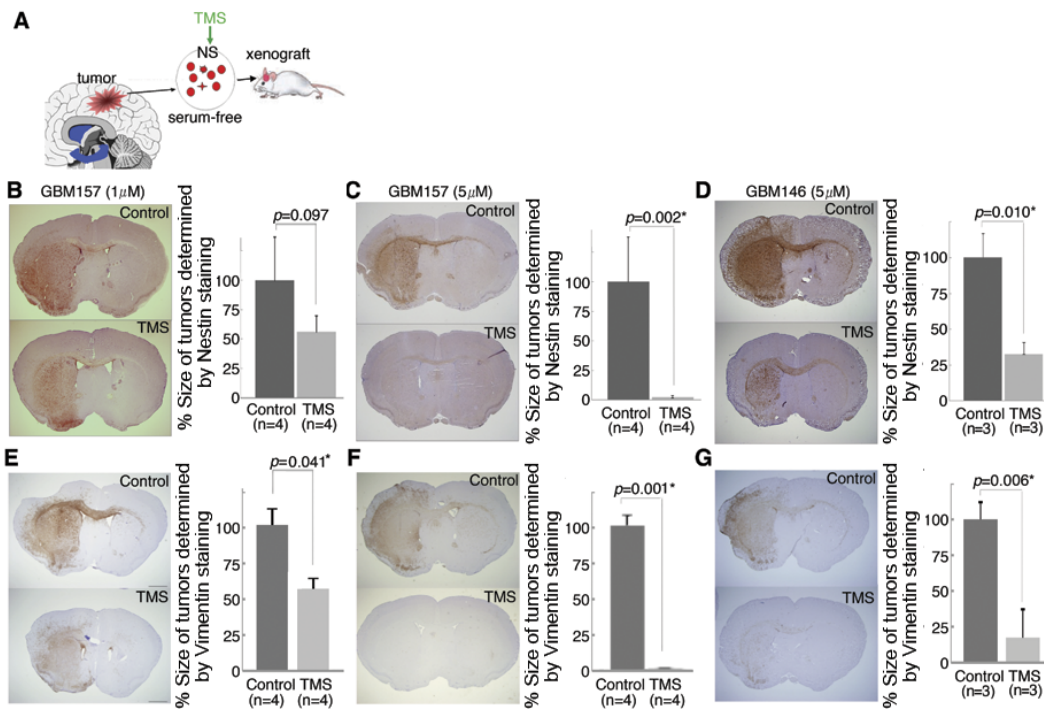


Fig. S5.

A, Each panel indicates staining with Annexin V or Propidium Iodide labeling of GBM sphere cells treated with either DMSO or TMS at indicated doses for 48 h. Insets indicate cells in phase bright view. **B**, Western blot with TMS treated GBM spheres (GBM146 and 157) using p53 antibody. GAPDH was used as an internal control.

Fig. S5

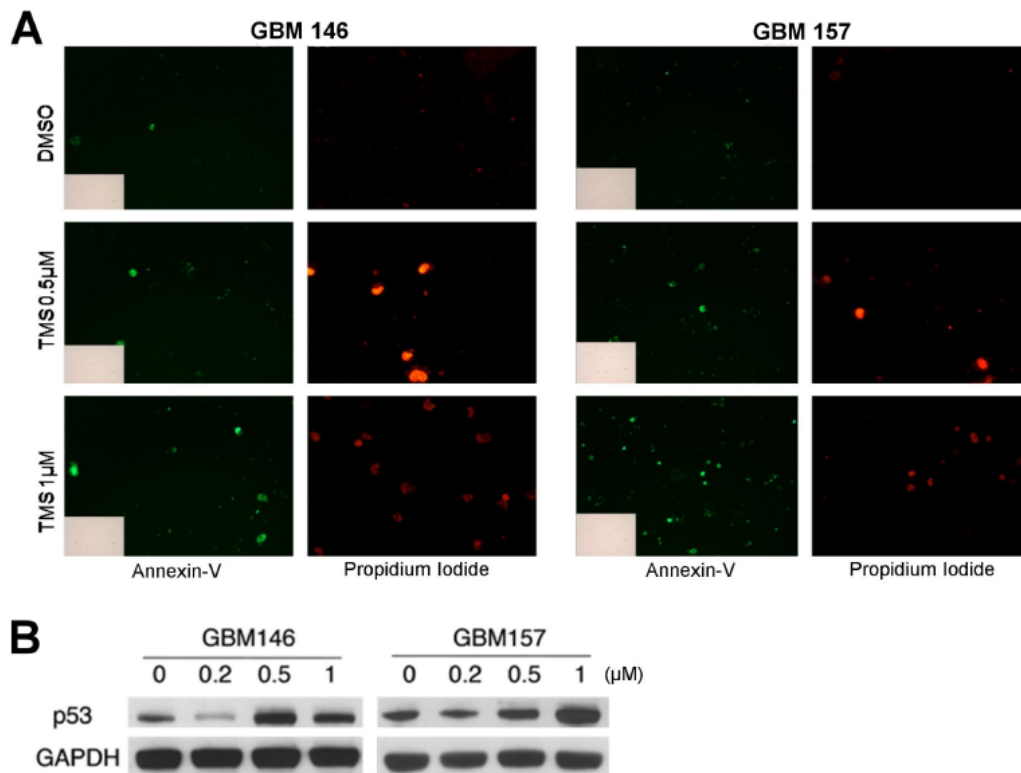


Fig. S6.

Western blot analysis of c-Myb expression in 4 GBM samples and 2 normal sphere samples after treatment with TMS at indicated doses (μM). GAPDH is used as internal control.

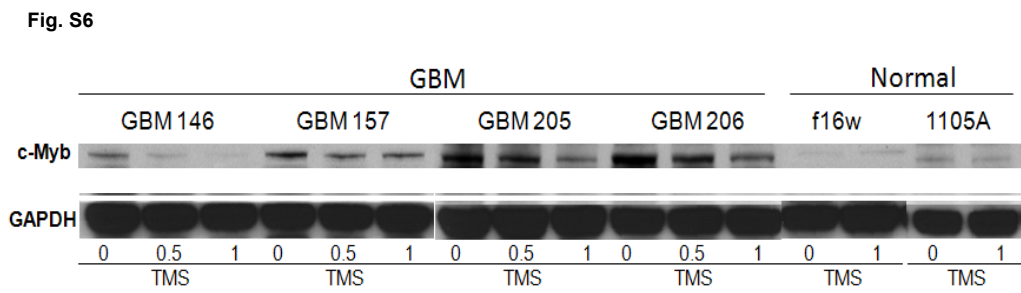


Fig. S7

Quantitative RT-PCR of c-Myb after overexpression of c-Myb or GFP (left) and knockdown of c-Myb (shc-Myb) or non-target control (shcontrol)(right) in GBM30 cells. Asterisk indicates statistical significance by (* $p < 0.05$).

Fig. S7

